Biocompatibility and Toxicological Effects of Doped, Functionalized and Pure Carbon Nanotubes

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14. ABSTRACT

We wrote a review article for Advanced Materials together with Dr. Hussain's group about the toxicity evaluation for safe use of nanomaterials. This paper has been highlighted by the journal as one of the most downloaded papers in two months (see report). From the experimental standpoint, we synthesized different types of carbon nanotubes (CNTs): multiwalled carbon nanotubes (MWNTs), nitrogen-doped (CNx) MWNTs, phosphorus- and nitrogen-doped (CNxPy) MWNTs, ethanol-based (carbonyl, carboxyl functionalized) CNT (COx-MWNTs) and B-doped (CBx) MWNTs. We also prepared and evaluated different suspensions for biological applications of CNTs. We found that H2SO4, acetic acid, HNO3/ H2SO4 mictures and chloroform were suitable for suspending the different types of tubes. Ag nanoparticles were chemically anchored on the surface of MWNTs, COxMWNTs and CNxMWNTs. Cellular function and immune response were evaluated to determine biocompatibility of the synthesized nanomaterials on the human keratinocyte cell line (HaCaT). Cellular assays revealed toxicity after 24h. However, full cellular recovery was observed at 48h. Therefore, Ag nanoparticles (if anchored to CNTs) have the ability to interact with cells and preserve cellular function.

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Synthesis, Carbon nanomaterials, Toxicity, Citotoxiciy, Biocompatibility, Ag Nanoparticles.

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1. ELABORATION OF A JOINT REVIEW PAPER FOR *ADVANCED MATERIALS*

In order to emphasize the importance of performing biocompatibility and toxicological tests on nanostructures, we decided to write an updated review on the field. This contribution was published in *Advanced Materials* (impact factor of 8) and has been highlighted by the journal as the most accessed article in two different months (February & April 2009). The review is also aimed to motivate multidisciplinary collaborations among biologists and materials scientists (see Fig. 1). The complete reference of this paper is: Hussain, S.M. Braydich-Stolle, L.K., Schrand, A.M., Murdock, R.C., Yu, K.O., Mattie, D.M., Schlager, J.J. and Terrones, M. (2008) "Toxicity Evaluation for Safe Use of Nanomaterials: Recent Achievements and Technical Challenges". *Advanced Materials (Review)* 21, 1-11.

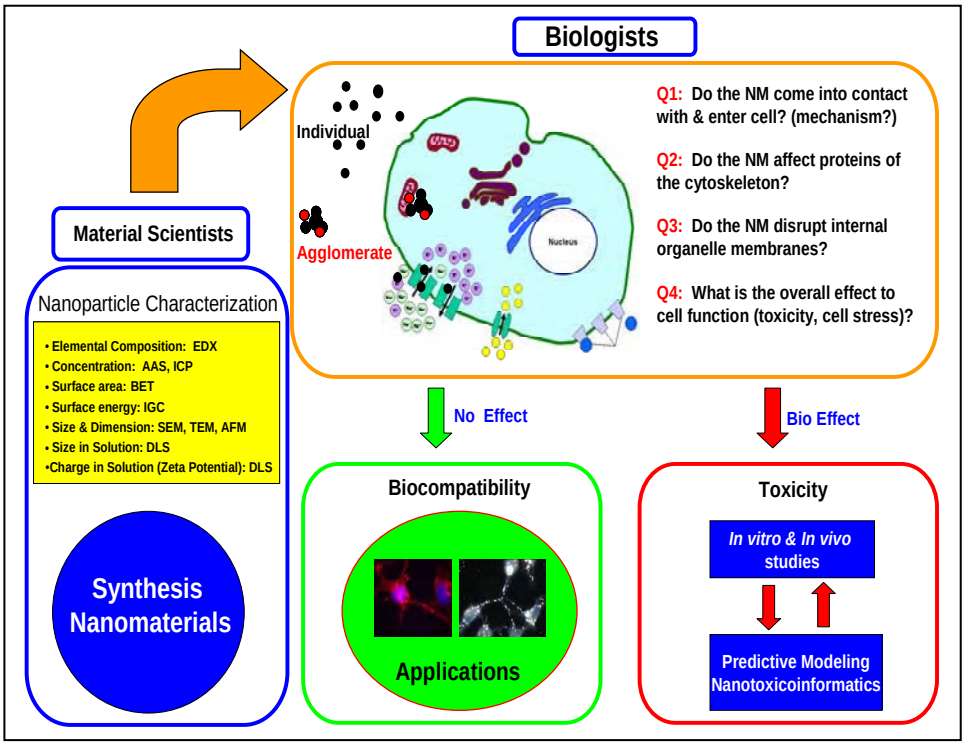

Figure 1.- Sketch showing the multidisciplinary approach for evaluating the biological responses of NMs and identifies the critical questions that researchers are focusing on answering.

2. PRODUCTION OF NANOSTRUCTURES

We have used the chemical vapor deposition method (CVD) for producing different types of multiwalled nanotubes (MWNTs). In order to do that, we set up a new sprayer system, equipped with an ultrasonic generator. This new system was capable of producing very clean nanotube samples, which becomes even more desirable when trying to assess cell viability and bicomopatibility of nanomaterials. Figure 2 shows a photograph of the experimental set up that we used for the nanotube synthesis. We produced pure carbon MWNTs, nitrogen-doped (CN_x) MWNTs, phosphorus- and nitrogen-doped (CN_xPy) MWNTs, ethanol-based (carbonyl, carboxyl functionalized) carbon nanotubes (CO_xMWNTs) and B-doped (CB_x) MWNTs. Some of these materials were used for producing uniform dispersions (see next section).

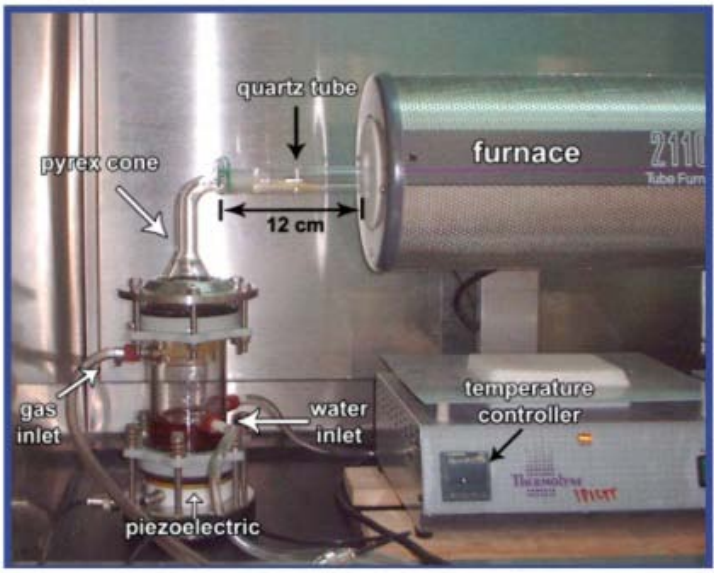

Figure 2.- Experimental setup installed at IPICYT for producing doped nanotubes.

CN_xMWNTs were synthesized by the thermal decomposition of a mixture of 2.5%wt of ferrocene ($FeCp_2$; 98% Aldrich®) and benzylamine (C_7H_9N; 99.9% Sigma-Aldrich®), follwing the method described elsewhere[13]. Briefly, solutions of ferrocene ($FeCp_2$) in benzylamine (C_7H_9N) were placed into the reservoir of the aerosol generator. The solution was subsequently turned into a mist by the action of a piezoelectric located at the bottom of the container. The mist flowed inside a quartz tube placed inside a tubular furnace, which was used as a substrate. The seed molecules dissociated when the mist reached the zone of the furnace at high temperature (850°C). Fe catalyst particles were formed and MWNTs grew perpendicular to the inner walls of the quartz tube. The whole process was carried out under an Ar atmosphere (Ar flow of ca. 3.5 l/min) for 15 min. The experimental parameters reported here are the optimized values that led to the highest yields of very clean CN_xMWNTs. Using this values, we were able to produce 2 gr of CN_xMWNTs in one day. Nitrogen was sucesfully incorporated within the hexagonal lattice of the nanotube walls, as revealed from energy-dispersive X-ray analysis (EDX) and X-ray photoelectron spectroscopy (XPS) analysis (not shown here).

Similarly, MWNTs were obtained by the decomposition of a solution of 2.5%wt of ferrocene ($FeCp_2$) and toluene (C_7H_8; 99% Fermont ®). The experimental parameters used were the same as those of the synthesis of CN_xMWNTs. For the production of CO_xMWNTs, a solution of ferrocene (2.5%wt), 1%wt of ethanol (CTR scientific®) and toluene (C_7H_8)[14] was used. For the syntesis of CN_xP_yMWNTs a precursor solution of benzylamine, ferrocene and triphenyl-phosphine P(C6H5)3

(TPP) (Sigma, 99%) was used. This solution was thermally decomposed using the same ultrasonic assisted CVD system at a temperature of 850°C.

CB_xMWNTs were succesfully synthesized using the same CVD method. For these experiments, we dissolved triethylborane ($C_6H_{15}B$) in mixture of toluene and ferrocene. The used temprature range was 750-960°C. Carefull caracterization of these samples is currently underway, in order to determine the amount of B incorporated in the MWNTs. Figure 3 shows representative scanning electron microscopy images of some of the nanomaterials synthesized during the project.

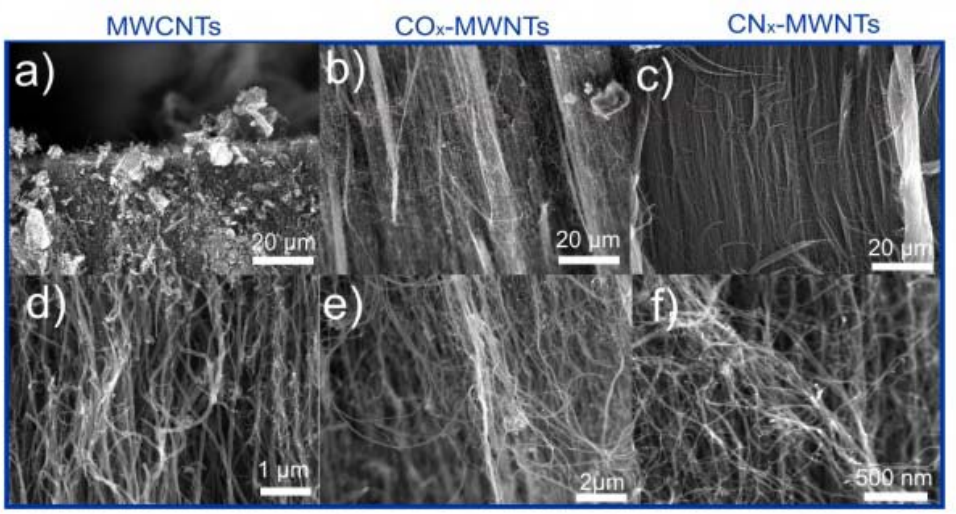

Figure 3.- SEM images of pristine nanotubes. (a,d) MWCNT. (b,e) CO_xMWCNTs. (c,f) CN_xMWCNTs

3. FABRICATION OF UNIFORM SUSPENSIONS

In order to perform an acurate assessment of the citotoxicity of nanomaterials, the preparation of stable suspensions becomes crucial. We performed suspension tests with MWNTs, CO_xMWNTs and CN_xMWNTs in different media. As solvents we used acetic acid, chloroform, acetone, H_2SO_4, NaOH, H_3PO_4, hexanes, different concentrations of HNO_3, ethanol, different mixtures of H_2SO_4/H_2O (1:1, 1:2, 1:3, 1:4

ratios) and different mixtures of HNO_3/H_2SO_4 (1:1, 4:1, 1:4 ratios). Concentrated H_2SO_4 was found to be optimal for producing suspensions of MWNTs, followed by acetic acid (67%). Figure 4 shows photographs of these optimal suspensions, right after one hour sonication and, later on, after 7 days.

Figure 4.- Photographs of MWNTs suspensions in two different solvents. The suspensions were placed in an ultrasonic bath for 1 hr. Right panels exhibit the suspensions after 7 days of ultrasonication.

CO_xMWNTs were succesfully suspended in a mixture of HNO_3/H_2SO_4 (1:4 ratio). Acetic acid and chloroform were found to be also suitable for suspending CO_x nanotubes (see Figure 5). Acetic acid at a pH of 0.6 was able to suspend CN_xMWNTs for more than one week (see Figure 6).

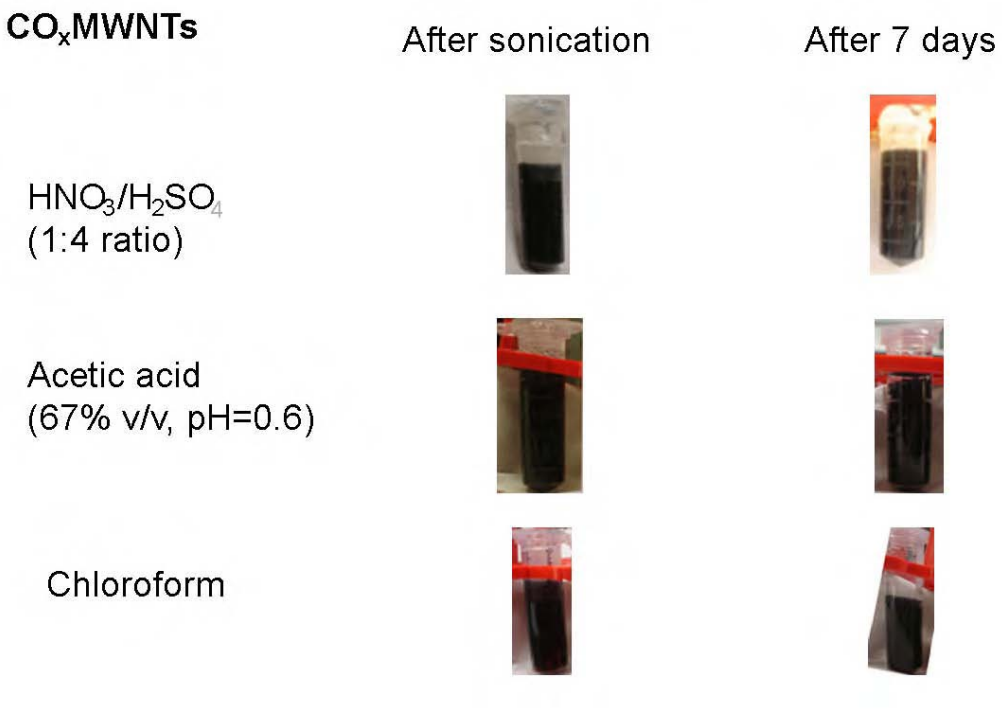

Figure 5.- Photographs of CO_xMWNTs suspensions in three different solvents. The suspensions were placed in an ultrasonic bath for 1 hr. Right panels exhibit the suspensions after 7 days of ultrasonication.

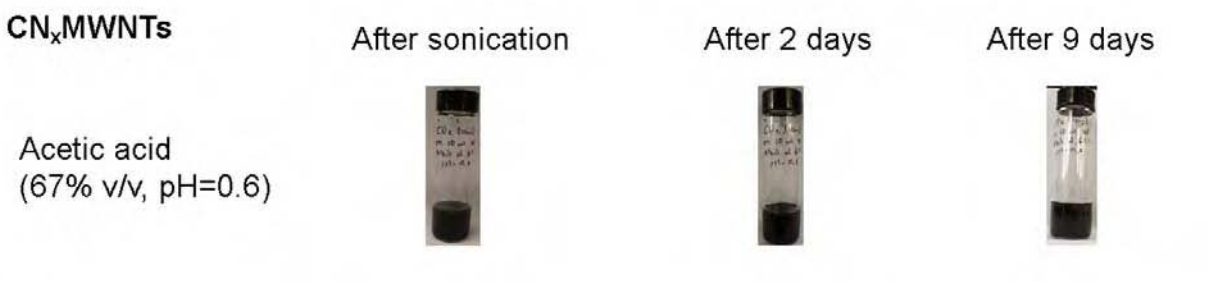

Figure 6.- Photographs of CN_xMWNTs suspensions in acetic acid. The suspensions were placed in an ultrasonic bath for 1 hr.

4. Toxicity Studies

Silver (Ag) nanoparticles were anchored on the surface of three different types of MWNTs. Therefore we synthesized three different types of Ag-MWNTs (pure carbon), Ag-MWNT-CO_x (carbonyl and carboxyl doped), and Ag-MWNT-CN_x

(nitrogen-doped). Cellular function and immune responses were evaluated to determine biocompatibility of the synthesized nanomaterials on the human keratinocyte cell line (HaCaT). Cellular assays revealed toxicity after 24h however, full cellular recovery was observed at 48h. These results suggest Ag nanoparticles can be anchored on MWNTs and have the ability to interact with the cells and preserve cellular function.

Background

Ag nanoparticles of extremely small size and large surface area exhibit different properties when compared to the bulk. Nanosized Ag possesses a high extinction coefficient, high surface plasmon resonance and anti-microbial properties which could be less toxic than the bulk form[1,2]. Currently Ag nanomaterials have a variety of uses in everyday consumer's lives such as: nano-Ag infused storage containers[3], nano-Ag coated surfaces of medical devices to reduce hospital related infections[4], bandages[5], footwear[6] and countless household items which claim to be anti-microbial. In the future, nano-silver's high surface plasmon resonance has a possibility for many color based biosensor applications and different lab-on a chip sensors. While all of these properties appear to make nano-Ag the new "wonder-drug" of the nanotechnology world, problems arise.

One of the key features of nanosilver is that it has been shown to be toxic in a size dependent manner, according to *Carlson et. al* [7]. The toxicity in the size dependent manner is shown to be due to reactive oxygen species (ROS) production. A decrease in the membrane integrity allows the leakage of ROS

outside the cell leading to apoptosis[7]. Another mechanism of Ag nanoparticles toxicity is believed to be due to the strong affinity of Ag to thiol groups[8]. Thiol groups are functional groups of the amino acid cysteine and are considered to be highly reactive. In an effort to make the silver less toxic silver nanoparticles were coated with polysaccharide[9]. Overtime, the coating dissolves in the body again rendering the cell to the toxic properties of the Ag nanoparticles. It has been reported that Ag-10-PS and Ag-15-HC cause the same decrease in cell viability after a six day exposure in neuronal cells[10]. If the coating is dissolving after just six days in the media, the time to become toxic could be much shorter in the human body[10].

In view of the extensive biomedical applications of Ag nanoparticles it is important to synthesize biocompatible Ag particles. One possible method for reducing the toxicity of Ag nanoparticles is by anchoring them on multi-walled carbon nanotubes (MWNT), which are easily functionalized and have been shown to be non-toxic depending on the impurities left in the sample[11]. MWNTs could be used as delivery vehicles due to their chemical structure and unique ability to be functionalized with various particles and chemical groups. Functionalization of single walled nanotubes (SWCNT) leads to the breakage of C=C bonds leaving “holes” in the structure causing significant changes in properties of the nanotubes. Unlike the SWCNTs, MWNTs do not have bond breakage in the inner tube shells, and therefore these “holes” do not have much influence in the mechanical properties of the tubes, but these “holes” are active and could be used as anchoring sites[12].

Materials and Methods

Pure MWNTs are promising for biocompatible interaction with human keratinocytes. This study had the goal of determine if the anchoring of Ag nanoparticles on MWNTs could be used in future to deliver Ag to the living cells non-invasively. We used three types of carbon nanomaterials: MWNTs, CN_xMWNTs and CO_xMWNTs. These materials served as substrates for the anchoring of Ag nanoparticles and were synthesized by CVD (for more information please refer to the first part of this report).

The three different types of nanotubes tested in this work were used without further modification (e.g. acid treatments). The general procedure consisted of adding 1mg of the nanotubes to a solution of acetone (C_3H_6O; 20 ml) and silver nitrate ($AgNO_3$; 83µl from a solution 0.1 N, J.T. Baker®) in a flask. Subsequently, the suspension was dispersed ultrasonically for one hour. The solution was then placed in a water bath to increase the temperature to ~56°C, and at this point 10 ml of N,N-dimethylformamide (DMF, 99% Sigma-Aldrich®) were added as a reducing agent; the temperature of the suspension was maintained for 20 additional minutes. Subsequently, the nanotube samples were centrifuged, washed with distilled water twice and dried at 70° C in an oven.

The characterization of the composites was carried out by SEM, using an FEI XL30 FEG/SFEG, operated at 15 kV. The X-ray powder diffraction studies were performed using an XRD D8 ADVANCE – BRUKER AXS, with a Cu K_α radiation (λ = 1.54060 Å). The operating current and voltage were maintained at 35 kV and 25

mA. High resolution images were taken with a HRTEM -field emission JEOL-JEM-3000F operating at 300 keV.

HaCaT cells were donated generously by Dr. James F. Dillman III, of the U.S. Army Medical Research Institute of Chemical Defense 3100 Ricketts Point Rd Aberdeen Proving Ground, MD 21010-5400. The HaCaT cells were maintained in RPMI-1640 with 1% Penicillin-Streptomycin and 10% FBS at 37.0°C and 5% CO_2[15]. The cells were passaged using Trypsin-EDTA and PBS when they reached 70-80% confluency. For nanomaterial exposures, RPMI media supplemented with 1% penicillin/streptomycin was used.

CytoViva imaging was used to determine the interaction between the Ag-nanotube systems and the cells as previously described[16]. Cell viability was assayed by MTS as indicated by the CellTiter 96®AQueous One Solution from Promega, which measures mitochondrial function and directly correlates to cell viability. The relative cell viability (%) related to control wells containing cell culture exposure media without nanoparticles was calculated by [A]test / [A] control x 100, where [A]test is the absorbance of the test sample and [A]control is the absorbance of control sample. Each experiment was done in triplicate.

The mitochondrial membrane potential was measured to examine changes in the mitochondrial membrane, which correlates to ROS production and apoptosis. The cells were exposed to 25µg/mL of Ag-nanotubes 24h. The protocol from the Mit-E-Ψ Mitochondrial Permeability Detection Kit (AK-116) was used throughout the assay. The cells were imaged using the confocal microscope and MMP was

measured based on images and computer software, which compares the fluorescent intensity of each well to the control.

Results

The nanotubes differed in chemical reactivity according to the thermogravimetric analysis performed. The reactivity observed can be summarized as follows: MWNTs < CO_xMWNTs < CN_xMWNTs. Figure 7 shows STEM images of the MWNTs/Ag composites and their particle size distribution. The MWNTs/Ag composite is presented in Figure 7-(**a-c**) with an average particle size of 6.64 ± 2.25 nm. Figure 7-(**d-f**) shows the CO_xMWNTs/Ag, and this composite presented a bimodal histogram in the Ag particle sizewith an average size of 11.75 ± 4.65 nm. Finally, Figure 7-(**g-i**) shows the CN_xMWNTs/Ag composite, this material also exhibited a bimodal behavior in the Ag particle, giving an average value of 13.25 ± 3.94 nm.

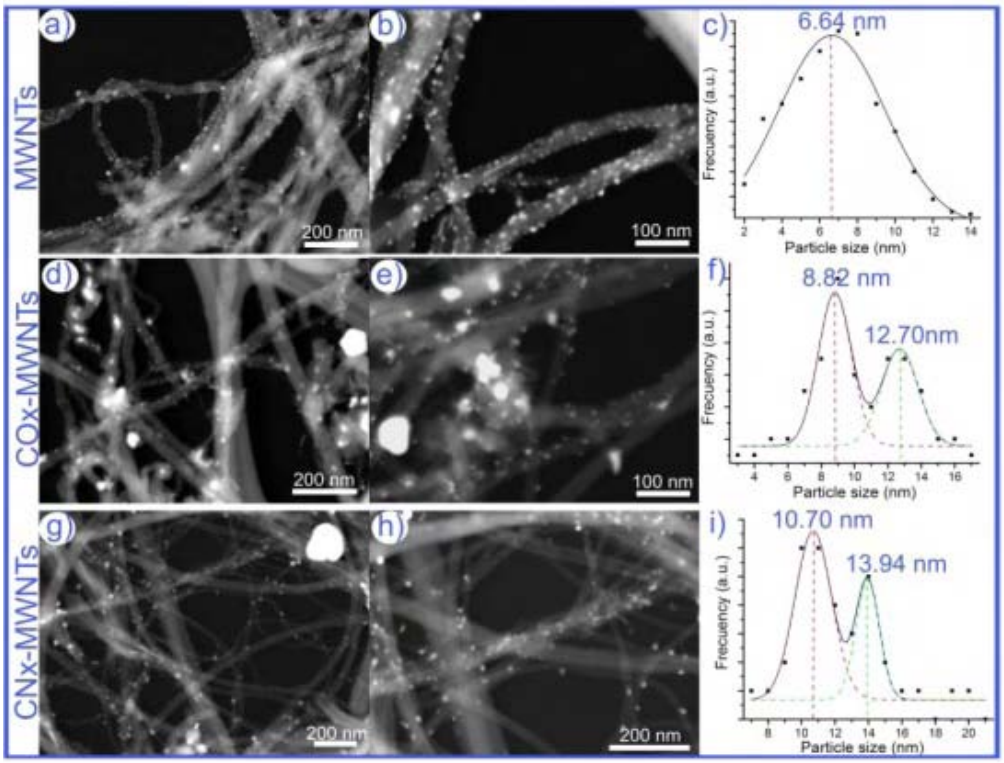

Figure 7.- SEM images of MWNTs (a,b); CO_xMWNTs (d,e) and CN_xMWNT (g,h). The Silver (Ag) particle size for each particle type of tube is also depicted: (c) for MWNTs is 6.64nm, (f). for CO_xMWNTs are 8.82nm and 12.70nm, and (i) for CN_xMWNTs are 10.70nm and 13.94nm.

The inflammatory response of the HaCaT cells to the nanotubes was measured using real-time PCR. HaCaT cells typically produce IL-6 and TNF-α, so these cytokines were chosen to be measured[17]. IL-6 and TNF-α primers were ordered from Integrated DNA technology (IDT) and used for PCR. Cells were plated at 300,000 cells/mL in 6 well plates and dosed with 25μg/mL of the different nanomaterials for 6h and 24h. After each time point the RNA was isolated using the Qiagen RNA isolation kit. The isolated RNA was then NanoDropped to determine the amount of RNA present in the sample. Then 1μg of RNA was used in real time PCR analysis. The Express Script from Invitrogen was used and 100nM forward and reverse primers for TNF-α, IL-6 and beta actin were used. On the Stratagen MX3005P the RNA was reverse transcribed for 30min at 50°C and then amplified using a cycle of 94°C for 30sec, 60°C for 1 min at 40 cycles. The TNF-α, IL-6 expression was normalized based on beta actin expression.

Changes in inflammatory response can provide information about the cellular environment and determine cellular stress. Figure 8 shows the PCR results at 6h, and illustrated slight increases in TNF-α and IL-6 when compared to the control. The MWNT-Ag, CN_xMWNT-Ag and CO_xMWNT-Ag all showed a small initial increase in IL-6 and TNF-α, but it was not considered significant. After 24h, the increase in IL-6 did not change in any of the treatment groups, but TNF-α continued to increase (Figure 8) in the MWNT-Ag and CO_x-Ag. This could be due to IL-6

stimulating the immune response of TNF-α, thus leading to inflammation, cell death and viral inhibition.

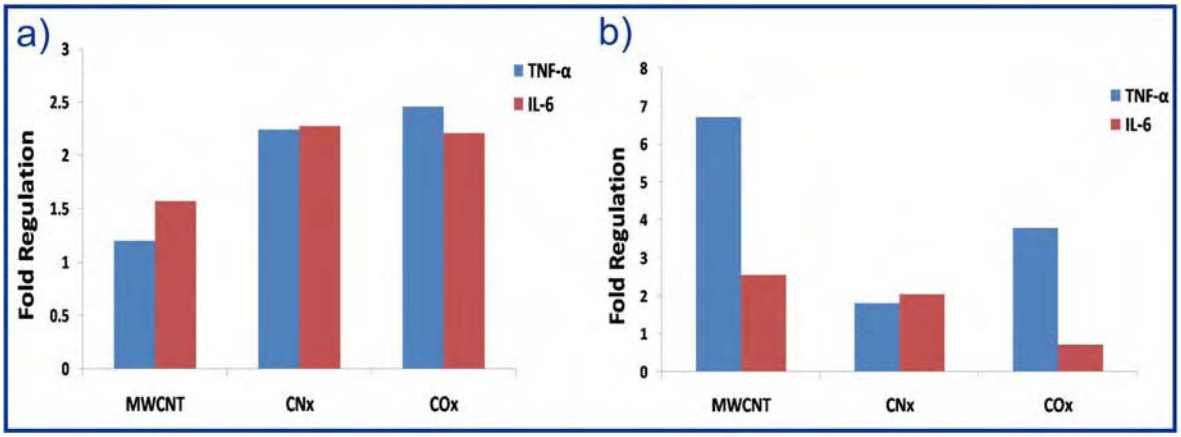

Figure 8.- Change in inflammatory cytokine expression following A.) 6h B.) 24h exposure to nanomaterials. PCR results looking at the inflammatory markers TNF-α and IL-6 in HaCaT cells show no significant increases at 6h for TNF-α or IL-6 and significant increase in TNF-α for Ag-MWNT and COx-MWNT.

The increase in TNF-α could activate the NF-κB pathway and result in transcription of genes involved in stress and inflammation. NF-κB can lead to apoptosis or cellular recover and due to recovery assays we performed we can hypothesize the cells are recovering[18]. Measuring the mitochondrial membrane potential at 24h after exposure showed a significant decrease in mitochondrial membrane potential (Figure 9A). The significant decrease in MMP indicated a decrease in cell viability. The decreases in MMP provide information that the cell is undergoing oxidative stress and possibly preparing to undergo apoptosis. Pictures taken with the confocal microscope showed the same qualitative results. The pictures showed more red fluorescence in the CN_xMWNT-Ag and CO_xMWNT than the control (Figure 9B), and also slightly more red fluorescence in the MWNT-Ag, indicating a reduction in mitochondrial membrane potential (Figure 9B). Control Cells showed

no red fluorescence, while the positive controls showed high amounts of red fluorescence indicating that the test was adequately performed .

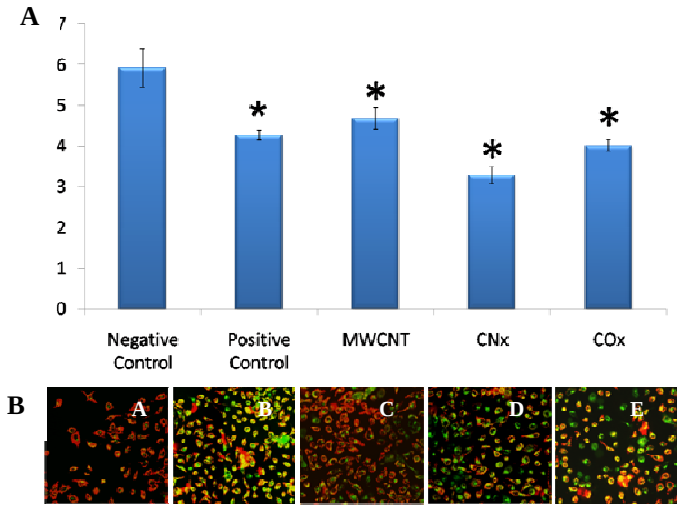

Figure 9.- The reductions in mitochondrial membrane function (MMP) after 24h of exposure to 25µg/mL of nanomaterials. A.)Change in mitochondrial membrane potential of nanomaterials treated cells compared to the untreated control and positive control dosed with hydrogen peroxide. B.) Qualitative Confocol images of HaCaT cells A.) Negative Control, B.) Positive Control, C.) MWNT-Ag, D.) CNx-MWNT-Ag, E.) COx-MWNT-Ag.

Mitochondrial membrane potential measures the amount of hydrogen ions pumped across the inner mitochondrial membrane during oxidative phosphorylation and the change in the amount of hydrogen pumped out is measured and related as the mitochondrial membrane potential. A decrease in the mitochondrial membrane potential also leads to the conclusion that the cells are responding to an oxidative stress.

Figure 10 shows the MTS results of the HaCaT cells exposed to varying concentrations of Ag-nanotubes. The cells have completely recovered from the exposure by the 48h timepoint (Figure 10). The decreases in cellular viability observed after 72h and 1 week could be caused by the cells having a large increase in viability at 48h and becoming over confluent, thus causing cell death.

Cell viability could also have decreased due to the cells being placed in the exposure media, which does not contain the FBS needed for normal cellular growth.

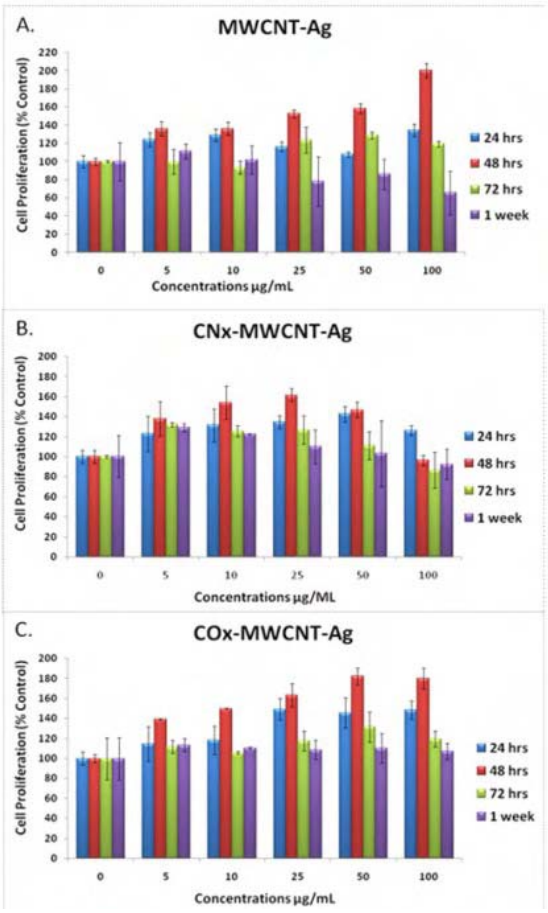

Figure 10.- Cell viability in HaCaT cells exposed to different carbon nanomaterials at varying time points A.) MWNT-Ag, B.) CNx-MWNT-Ag, C.) COx-MWNT-Ag.

Discussion

MWNTs have a potential use as an imaging agent or as a biosensor. Due to their near infrared emission they are able to respond to local dielectric function but still remain stable[19]. MWNTs also have the ability to be doped with different functional groups providing more hope for their future use in these fields. Detection using MWNTs would allow us for greater tissue penetration and reduce fluorescent background. Imaging would last for under 24h and therefore would be considered

an acute exposure. Therefore, and based on our results, we would expect to observe an immediate inflammatory response, but the body could recover from this exposure. With the cells being able to recover, this technique could be used in future imaging procedures. It is also important to mention that these Ag-tube systems do not contain any thiol group, and further studies with Ag nanoparticles with thiol groups should be carried out. Our study examined the biocompatibility of MWNTs coated with Ag nanoparticles, a known anti-microbial agent, and doped with nitrogen and carbonyl groups to increase the biocompatibility.

While the cells show this initial inflammatory response, we observe that they also have an ability to recover over time, which leads us to hypothesize the activation of NF-κB is leading to recovery. The inflammatory response must help the cells to recover from the exposure rather than send the cells directly into apoptosis. The results of the MTS a 24h, 48h, 72h and 1 week reveal that the cells show an increase in viability over time and thus recovery. The recovery period occurs in as little as 48h for each of the exposures. After the 48h time point we do observe a decrease in cellular viability, but this is not believed to be due to the presence of the nanomaterials. This is possibly due to the cells becoming too confluent in the 96 well plate, and being in the exposure media for long periods of time. Figure 11 shows all three types of MWNTs coated with Ag entered the cells, and were bound to the nucleus or nuclear region based on the CytoViva imaging. We could also determine the binding due to the high concentration of MWNTs in the images taken after 1 week. The cells had been washed and placed in exposure media for a week. The human keratinocytes appeared to exhibit some actin filament disruption

caused by the Ag-MWNTs. However, the MTS results showed no toxicity, but rather an increase in cell viability. Some of the actin filament disruption could be due to the exposure media, because the cells do not grow normally due to the lack of FBS. This is also indicated in the control cells, which appear to become rounded over time.

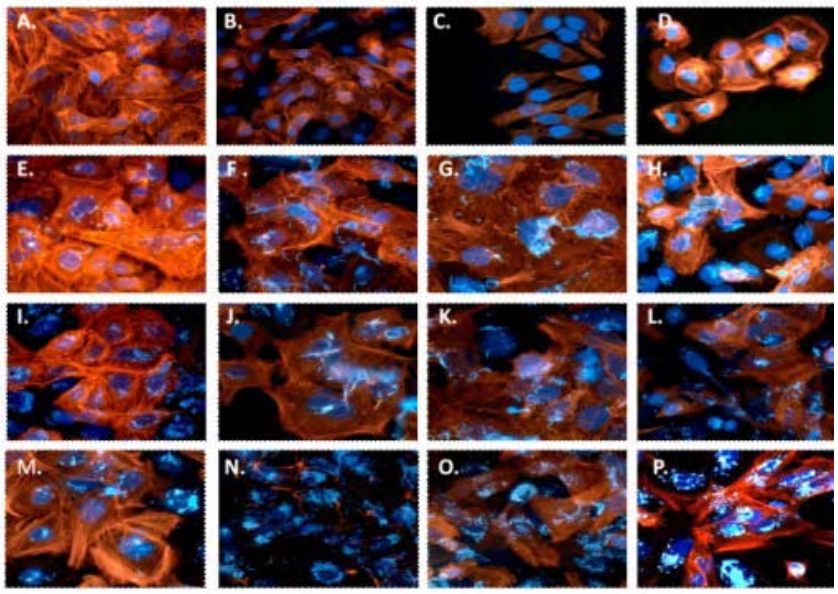

Figure 11.- CytoViva imaging of HaCaT cells dosed with nanomaterials and then stained for actin Control HaCaT cells at A. 24h, B. 48h, C. 72h and D. 1 week Ag-MWNTs at E. 24h, F. 48h, G. 72h and H. 1 week. Ag-CNx at I. 24h, J. 48h, K. 72h and L. 1 week and Ag-COx M. 24h, N. 48h, O. 72h and P. 1 week Each picture with each particle type shows localized nuclear binding with the nucleus stained blue and the actin filaments stained red/orange. The particles appear as the bright spots. There appears to be limited actin filament disruption in comparison to the control pictures. Binding can be determined based on the number of particles seen even after the 1 week time period in the exposure media.

The ability of human keratinocytes to recover after an acute exposure demonstrates these Ag-nanotube systems still have a possibility for being used as delivery systems even, with an initial toxicity. Table 1 shows the summary of the acute exposure to the nanomaterial composites.

Ag nanoparticles have shown to be toxic in a size dependent manner. In particular Ag-15 has been demonstrated to be toxic to BRL-3A cells, C18-4 cell, and macrophages. Ag-15 has been shown to be toxic regardless of the coating, which was in agreement with the results that Ag toxicity increases with size reduction[9,10]. Silver is the most toxic at small sizes due to its ability to produce more ROS at the smaller sizes.

Table 1. Summary Table showing particle size, cellular morphology, change in mitochondrial membrane potential, inflammatory response and recovery

Particle Type	Ave Diameter (nm)	Cell Morphology	MMP	PCR 6h fold increase		PCR 24h fold increase		MTS Recovery Cell Proliferation
				IL-6	TNF-α	IL-6	TNF-α	
Ag-MWNT	6.64	Minor Actin Disruption, Nuclear binding	* decrease	1.5	1.3	2.7	6.8	Increase
Ag-CNx-MWNT	8.82 & 12.70	No Disruption, Nuclear binding	* decrease	2.4	2.4	2	1.9	Increase
Ag-COx-MWNT	10.70 & 13.94	No Disruption, Nuclear binding	* decrease	2.6	2.5	.5	4	Increase

The Ag nanoparticles used in this study were all under 30nm and should have been toxic in a size and dose dependent manner. When comparing ROS production and cell viability between previous studies and our study at the same concentrations and time points, we observed a much greater increase in biocompatibility in the Ag nanoparticle anchored on any type of MWNTs rather than isolated Ag nanoparticles.

All three carbon nanotubes were coated with Ag, but interestingly those Ag systems containing the CN_xMWNTs and CO_xMWNTs did not increase

biocompatibility. MWNTs with nitrogen and carbonyl groups produced a greater reduction in mitochondrial membrane potential versus the pure carbon MWNTs (CN_x>CO_x>MWNT). Nitrogen and the oxygen from the carbonyl group could be forming ROS products within the cell. The functional groups could also be interacting with proteins or cell structures. These interactions could lead to the inhibition of cellular processes causing the reduction in cell viability. While they do cause the reduction in mitochondrial membrane potential, the presence of MWNTs first creates an inflammatory response to protect cells, but then they are responsible of repairing the human keratinocytes.

In a study performed by Cheng et al[20], the acute and long term effects of functionalized MWNTs were examined in zebrafish. In the early stages, the zebrafish generated an immune response. The zebrafish that were exposed to the MWNTs produced an initial response, but the nanotubes were cleared from their body after 96h. However, the second generation of the zebrafish had lower survival rate suggesting that MWNTs had no significant toxicity initially but did display long term effects on the progeny. These results agree with ours showing a significant inflammatory response followed by the cells capacity for recovery.

Nanomaterials hold the key to several unopened doors in the science field, but several questions still need to be answered. Is the possibility of a toxic reaction worth the risk? Further biocompatibility assays need to be performed before Ag-nanotube systems can be considered for medical or biological uses. If we can functionalize carbon nanotubes to be biocompatible the possibilities are endless.

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REVIEW ARTICLE

Toxicity Evaluation for Safe Use of Nanomaterials: Recent Achievements and Technical Challenges

By *Saber M. Hussain,* Laura K. Braydich-Stolle, Amanda M. Schrand, Richard C. Murdock, Kyung O. Yu, David M. Mattie, John J. Schlager,* and *Mauricio Terrones**

Recent developments in the field of nanotechnology involving the synthesis of novel nanomaterials (NM) have attracted the attention of numerous scientists owing to the possibility of degradative perturbations in human health. This Review evaluates previous investigations related to NM toxicity studies using biological models and describes the limitations that often prevent toxicologists from identifying whether NM pose a real hazard to human health. One major limitation to assess toxicity is the characterization of the NM prior to and after exposure to living cells or animals. The most relevant physicochemical characteristics of NM are: size, surface chemistry, crystallinity, morphology, solubility, aggregation tendency, homogeneity of dispersions, and turbidity. All of these properties need to be assessed in order to determine their contribution to toxicity. Due to the lack of appropriate methods to determine the physicochemical nature of nanoparticles in biological systems, the exact nature of NM toxicity is not fully described or understood at this time. This Review emphasizes the need for state-of-the-art physicochemical characterization, the determination of appropriate exposure protocols and reliable methods for assessing NM internalization and their kinetics in living organisms. Once these issues are addressed, optimal experimental conditions could be established in order to identify if NM pose a threat to human health. Multidisciplinary research between materials scientists and life scientists should overcome these limitations in identifying the true hazards of NM.

1. Introduction

Over the last decade, newly synthesized nanomaterials (NM), exhibiting a single dimension in the range of 1–100 nm,[1,2] have been found to exhibit fascinating physicochemical properties. In addition to their small size, in comparison to their bulk counterparts, NM have an increased surface area and reactivity, which allows them to easily translocate cell membranes, efficiently bind molecular species, and catalyze chemical reactions. In accordance with their enhanced properties, NM have been proposed for use in the fabrication of various devices such as catalysts, sensors, composite fillers, actuators, transistors, drug and gene deliverers, biosensors, virus inhibitors, and protein immobilizers. However, massive quantities of NM would need to be produced for these applications to be realized thereby increasing the potential risk of human exposure and raising additional concern about their short and long-term toxicological effects. Therefore, a necessary first step in assessing NM safety is to establish reliable sources of NM, define and accurately characterize NM properties of interest, and understand the significance of NM interactions with relevant biological systems. However, prior to NM introduction into aqueous media for nanotoxicity studies, the optimization of synthesis techniques to produce the *same* materials, account for variations in NM properties, and quality control procedures must be in place.

An explanation of the different physical characteristics, such as size, morphology, surface chemistry, and biological coatings, which results from the variety of synthesis techniques need to be understood in a biological context in order to improve the existing methods and develop new assays with appropriate quality-assurance controls at both the academic research and manufactured engineering levels. Pre-analytical errors could originate during the manufacturing stage and researchers should not solely rely on data from the production industry or assume that NM remain unchanged throughout the duration of experiments. Additional variations in NM properties from different batches or sources can lead to erroneous results. For example, if two nanosized powders (e.g., silver and carbon) are produced by two companies employing different synthesis techniques, the following questions arise: are the powders similar enough to be considered the same? If not, which characteristics are similar,

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which characteristics vary from the other, and what accounts for these differences? An additional point to consider is whether or not the NM sizes can be manufactured with a uniform or narrow size distribution. These factors complicate the comparison of NM from different companies, thus limiting the supply of characterized materials to one or a few manufacturers, and make it very difficult to compare lab-to-lab biological effects. Fortunately, general methods used to characterize such NM in biological systems could answer these questions and allow us to evaluate changes in terms of biological interactions.

The issues discussed above reveal the need to establish comprehensive characterization of the NM being examined in nanotoxicity studies. The properties of NM that could potentially contribute to toxicity include: size, morphology, mass, composition, crystallinity, surface area, surface chemistry, reactivity, thermal response, and energetic behavior. These properties raise questions concerning adequate dosimetry and accurate methods for detecting NM in biological tissues. Additionally, standard reference materials (SRMs) need to be defined to aid in NM characterization and allow an accurate lab-to-lab comparison. Currently, SRMs are being developed by the National Institute of Standards and Technology (NIST) and the National Institute for Occupational Safety and Health (NIOSH) for physical and chemical characterization including gold NMs (sizes ranging from 1 to 100 nm) and other elemental thin films and single-phase nanoscale particles.[3] The only existing NMs with SRMs are polystyrene spheres in water suspensions[4] and some types of carbon nanotubes.[5] However, there are no standard cell lines or models in which these materials have been consistently tested, which leads to apparent discrepancies in the actual toxicity of similar materials and no concrete conclusions regarding NM safety.

Collaboration between materials scientists and life scientists is critical in making progress in this rapidly growing field. First, discussions regarding the synthesis techniques used by materials scientists and the resulting NM properties need to be understood from a biological perspective regarding their reactivity, dispersion, persistence, and solubility. The preliminary goals could include improved, environmentally friendly ways to synthesize NM, greater reproducibility and uniformity of NM characteristics such as size, morphology, surface chemistry, and the development of SRMs. From basic research to applications, the surface properties of an NM must be carefully characterized and evaluated in relevant models. The dynamic nature of NMs in biological systems is of particular importance as their properties may change depending on the environment (e.g., the pH), transport, and mechanisms of accumulation, degradation, and release. Finally, more appropriate animal studies and modeling of multiple systems and routes of NM exposure, and translocation in the human body will be necessary to realistically assess the risk of NM. Figure 1 illustrates the multidisciplinary approach, which is essential for effectively evaluating the biological responses of NM, and identifies the critical questions that researchers are focusing on answering.

Therefore, the focus of this Review will be to summarize briefly the important results of nanotoxicity studies conducted to date with an emphasis on NM characteristics that are suspected to contribute to their toxic potential as well as pitfalls and limitations that should be addressed by both materials scientists and life scientists. A description of the current in vitro analysis techniques and materials characterization methods will be discussed within the context of the ongoing challenges and recommendations will be made for achieving more reliable measures of NM safety.

2. Nanotoxicity Overview

2.1. Target Systems, Organs, and Cells

Several in vivo and in vitro studies have demonstrated that NM could induce toxicity in multiple organ systems. A large portion of the studies have focused on lung exposure because inhalation is a major source of higher-dose exposure and, ultimately, systemic absorption. Since real exposure may occur by accidental ingestion, absorption through the skin, inhalation or by systemic dosage for diagnostic and therapeutic purposes, it is important to evaluate NM in other systems in addition to lungs. Both laboratory-generated and ambient NM are capable of producing pulmonary inflammation, and can also affect other organs by passing through the lung epithelium and reaching interstitial tissues.[6–8] This effect seems

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